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EPIGENETIC MODIFYING COMPOUNDS AFFECT THE ACTIVITY OF PRIMARY HUMAN ARTICULAR CHONDROCYTES AND MESENCHYMAL STEM CELLS UNDERGOING CHONDROGENESIS

S. Snelling, A. Kramm, P. Cain, C. Yapp, A. Carr, A. Price, U. Oppermann. *Univ. of Oxford, Oxford, United Kingdom*

Purpose: Epigenetic regulation of cell behavior is important in both chondrogenesis and in the homeostasis of chondrocytes. Epigenetic changes have also been identified in OA chondrocytes. Compounds that can regulate chondrogenesis and primary human articular chondrocyte (HAC) behavior could thus be important in tissue engineering and in regulation of OA development. Our library of epigenetic modifying compounds are both specific and broad-spectrum and targets include methyltrasnferases, histone deacetylases, histone protein kinases and bromodomains. In this preliminary study we have screened for the effect of these compounds on cell viability, cell number and ECM production

Methods: Human mesenchymal stem cells (MSCs) were induced to undergo chondrogenesis and epigenetic modifying compounds were added on either day 1 or day 7. Primary HAC were obtained from the damaged and undamaged regions of the femoral condyle from individuals undergoing total knee replacement for OA. Following 4 days incubation with the epigenetic modifying compounds cell viability was measured using Prestoblue. Cells were then fixed and cell number assessed using DAPI staining, and ECM production assessed using Alcian blue staining and Nile red incorporation

Results: Epigenetic modifying compounds altered cell viability, cell number and ECM production in HAC and during chondrogenesis of MSCs. Compounds including Methylstat, an inhibitor of the Jumonji C domain-containing histone trimethyl demethylases caused an increase in the viability:cell number ratio in MSCs and damaged and undamaged HAC, whilst 5-iodotubercidin increased viability:cell number in MSCs only. GSK-J4, which blocks H3K27 demethylation reduced viability and cell number in MSCs and damaged and undamaged HAC. However there was an increase in the viability:cell number ratio in damaged chondrocytes compared to control, whilst undamaged chondrocytes showed a relative decrease in this ratio. ECM accumulation in chondrogenesis was reduced by compounds including GSK-J4 and increased by the PARP inhibitor Rucaparib

Conclusions: This preliminary screen demonstrates the utility of epigenetic modifying compounds in the regulation of chondrogenesis and HAC behavior. The differential effects of compounds in HAC and MSC indicates the importance of fine regulation of epigenetic activities for controlling chondrogenesis, and understanding and modifying the processes that may be disrupted during OA pathogenesis.

Future work will use an expanded library of compounds and will further explore the impact of epigenetic modifiers on chondrocyte viability, ECM production and the expression and activity of chondrocyte and OA-relevant genes.

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CULTURING TEMPERATURE AFFECTS CHONDROCYTE DIFFERENTIATION AND EXTEACELLULAR MATRIX FORMATION

A. Ito[†], T. Aoyama[‡], J. Tajino[†], M. Nagai[†], S. Yamaguchi[†], H. Iijima[†], X. Zhang[†], H. Akiyama[§], H. Kuroki[†]. [†]*Dept. of Motor Function Analysis, Graduate Sch. of Med., Kyoto Univ., Kyoto, Japan;* [‡]*Dept. of Dev. and Rehabilitation of Motor Function, Graduate Sch. of Med., Kyoto, Japan;* [§]*Dep. of Orthopaedic Surgery, Graduate Sch. of Med., Kyoto Univ., Kyoto, Japan*

Purpose: The intrinsic capacity of cartilage to repair articular chondral defects is poor. Autologous chondrocyte transplantation (ACI) technique has been already used for clinical treatment. However, it has been reported that primary cultured chondrocytes dedifferentiate to a fibroblast-like state when cultivated in monolayer condition. This shift in cellular differentiation has been demonstrated both by morphological changes and by alterations in some chondrocyte specific gene expression patterns. While many studies, investigating the effect of growth factor and scaffolds on the cellular differentiation, have been reported, it is still unknown whether the culturing temperature affects on the cellular differentiation, chondrocyte specific expression patterns and its extracellular matrix (ECM) formation. The fact that 37°C is generally selected as the culturing temperature for in vitro studies, not 32°C, which is equivalent to the normal intra-joint temperature implicates that the culturing temperature might have a crucial role for preventing cellular dedifferentiation not only in vitro but also in transplanted tissues. The purpose of this study is to investigate the effects of the different culturing temperature on chondrocyte differentiation and ECM formation.

Methods: Primary articular chondrocytes were isolated from porcine knee joint. Culturing temperatures were set at following 3 condition, 32°C as around normal intra-joint temperature, 37°C as deep body temperature and 41°C as around threshold temperature of mammal cell survival. Cell viability was measured by trypan blue dye exclusion test in 48 and 96 hours, and TUNEL assay in 78 hours. To assess the morphological changes, chondrocytes cultivated at each temperature for 48 hours were drawn their contour on the ImageJ manually, and then measured its average area and aspect ratio. Gene expression changes of a monolayer and a pellet cultured chondrocytes were analyzed by real time RT-PCR. To investigate the effects on the ECM formation, tissue sections were made and GAG synthesis was analyzed by alcian blue staining. GAG contents were quantified by DMMB assay.

Results: There are no significant differences in cell viability (over 90 % viability) and apoptosis ratio (under 1 % positive ratio in TUNEL assay) between the culturing temperatures. The chondrocyte aspect ratio was significantly higher in higher temperature, while there is no significant difference in the area between the culturing temperatures. The expression of COL2/COL1, Aggrecan and SOX9 were increased in temperature-dependent manner both in monolayer and in pellet culture. From the alcian blue staining, the synthesis of GAG was observed in 37°C and 41°C in the limbus of the pellet, while not in 32°C. GAG content quantified by DMMB assay was highest in 41°C.

Conclusions: The results of the morphological assessment suggested that the higher temperature induced the fibroblast-like formation which indicates the promotion of the dedifferentiation. Nevertheless, chondrocyte specific gene expression patterns; like COL2, Aggrecan and Sox9 were increased in temperature-dependent manner which indicates the prevention of the dedifferentiation. ECM formation from both histological and biochemical analysis suggested that the higher temperature could enhance the synthesis of GAG. Taken together, we could assume that the culturing temperature has some role in cellular differentiation. Further studies will be needed to elucidate the details of the effects and mechanisms, with which the culturing temperature alters the chondrocyte properties.

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OXIDATIVE STRESS ACTIVATES APOPTOSIS SIGNAL-REGULATED KINASE (ASK1) IN ARTICULAR CHONDROCYTES

G. Eaton, C. Diallo, T. Freeman. *Thomas Jefferson Univ., Philadelphia, PA, USA*

Purpose: Apoptosis signal-regulated kinase (ASK1) is a MAP kinase, kinase, kinase that is involved in a wide range of cellular processes including stress-related responses, cytokine and growth factor signaling, cell cycle control and apoptotic cell death. However, the role of ASK1 in normal and pathological function of articular chondrocytes has not been investigated. To determine the role of the ASK1/p38/JNK MAP kinase pathway in articular chondrocytes, we studied ASK1 expression and activation of p38/JNK. Sustained activation of p38 is known to be required for chondrocyte differentiation and how ASK1 influences this activation during the process of osteoarthritis has not been shown.

Methods: To characterize the role of ASK1/p38/JNK MAP kinase pathway in articular cartilage changes, we initially evaluated ASK1, phoshorylated p38 and phosphorylated JNK levels by immunohistochemistry and confocal microscopy in human articular cartilage from discarded tissue from total knee arthroplasty surgery. We also investigated knee joints from aging ASK1 knockout mice and their wild type littermates. To assess the role of stress on ASK1/p38/JNK MAP kinase pathway in chondrocytes, known activators of ASK1; H2O2, TNF-α and calcium, as well as inhibitors, were added to both N1511 chondrocyte cell line and cultured mouse metatarsals and performed immunohistochemistry and western analysis.

Results: Articular chondrocytes express increased amounts of activated ASK1/p38/JNK protein in areas of articular cartilage undergoing early osteoarthritic changes. Our results on stress activation of N1511 chondrocytes in vitro show differential activation of this pathway in immature versus mature chondrocytes. In these cells, activation of ASK1

by oxidative stress (H2O2) or TNF-α promotes chondrocyte maturation and increases MMP-13, alkaline phosphatase and Col X expression. Inhibitors of ASK1 or p38 abolish the induction of these markers. We propose that the ASK1/p38/JNK MAP kinase cascade is an important regulator of chondrocyte differentiation and is increased during osteoarthritis. Taken together, our results show that chondrocyte maturation and death could be controlled by regulating the ASK1 MAPK signaling pathway. Further, inhibitors of this pathway would be useful for the prevention of chondrocyte maturation, for instance, in the treatment of osteoarthritis.

Conclusions: In conclusion, the MAP Kinase pathway has been implicated as a major player in chondrogenesis. Ask1 is a MAP kinase, kinase, kinase that activates both JNK and p38. To clarify the specific conditions under which each would be activated by Ask1 we looked at known activators of Ask1 and used immunofluorescence and western analysis to monitor the resultant effects on Ask1, JNK and p38. The activation of Ask1 seems to be consistent in both differentiated and undifferentiated N1511 chondrocytes. However, the resultant activation of JNK and p38 is differentiation and stimuli dependent. Inhibition of ASK1 appears to slow articular cartilage degeneration in mice.

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PTEN REGULATES THE EXPRESSIONS OF TYPEII COLLAGEN AND AGGRECAN UNDER OXIDATIVE STRESS IN HUMAN CHONDROCYTES

K. Iwasa, S. Hayashi, T. Fujishiro, K. Kawakita, N. Kanzaki, S. Hashimoto, N. Chinzei, S. Sakata, R. Kuroda, M. Kurosaka. *Kobe Univ. Graduate Sch., Kobe, Japan*

Purpose: Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) was identified as an important tumor suppressor gene. The function of PTEN is one of negative regulator of phosphoinositol-3-kinase (PI3K) signaling. PI3K pathway is critical for cell survival, differentiation and matrix synthesis. Othteoarthritis (OA) is one of the most common diseases in elderly people. Oxidative stress is considered to be one of critical factors for OA onset and progression. It is known that the expressions of PTEN are increased in OA chondrocytes, but the function is not clear. Therefore, we investigated the function of PTEN in human chondrocytes under oxidative stress.

Methods: OA cartilage samples were obtained from the patients undergoing total knee arthroplasty. Normal cartilage samples were obtained from patients of femoral neck fractures who underwent femoral head replacement surgeries to compare with OA chondrocytes. The expressions of PTEN between normal and OA chondrocytes were analyzed by real-time PCR. Then, normal chondrocytes were transfected with PTEN siRNA(200μM). After transfection of PTEN siRNA, chondrocytes were treated with insulin-like growth factor 1 (IGF-1)(50nM) and/or tert-butyl hydroperoxide (*t*-BHP)(200nM) which caused oxidative stress. Then, the expressions of PTEN, typeII collagen (Col2al), aggrecan (AGC) were analyzed by real-time PCR and the phosphorylations of Akt and ERK1/2 were analyzed by western blotting.

Results: The expressions of PTEN were remarkably increased in knee OA chondrocytes than in normal and hip OA chondrocytes (Figure1). The expressions of Col2al and AGC were decreased after treated with *t*-BHP (Figure2). The expressions of Col2a1 and AGC were increased after transfection of PTEN siRNA even when the oxidative stress was induced by *t*-BHP (Figure3). The phosphorylation of Akt and ERK1/2 were increased after transfection of PTEN siRNA (Figure4). The expressions of PTEN were remarkably increased in knee OA chondrocytes than in normal and hip OA chondrocytes (Figure1). The expressions of Col2al and AGC were decreased after treated with *t*-BHP (Figure2). The expressions of Col2a1 and AGC were increased after transfection of PTEN siRNA even when the oxidative stress was induced by *t*-BHP (Figure3). The phosphorylation of Akt and ERK1/2 were increased after transfection of PTEN siRNA (Figure4).

Conclusions: PTEN is one of the most important tumor suppressor genes like p53. We have reported that p53 was increased by shear stress, but PTEN was not increased (data not shown). The function of PTEN depends on its abundant expression. However, p53 acts emergently when DNA is damaged. The role of PTEN in human chondrocytes might be different from p53. In this study, we investigated the relationship between PTEN, COL2 and AGC expressions in human chondrocytes in response to oxidative stress. Expressions of COL2 and AGC were decreased by *t*-BHP, but increased when PTEN was inhibited by siRNA transfection. It is known that PI3K/Akt pathway is critical for cell survival, differentiation and matrix synthesis. PTEN inhibits PI3K/Akt strongly. Therefore, the inhibition of PTEN expression increased typeII collagen and agggrecan expression in response to oxidative stress. PTEN expressions in OA chondrocytes were much more increased than in normal chondrocytes, so it might be possible to increase matrix synthesis in OA chondrocytes by inhibition of PTEN even when oxidative stress is loaded.

In conclusion, we investigated the PTEN's role in human chondrocytes and we found that the expressions of COL2 and AGC under the oxidative stress were regulated by PTEN.

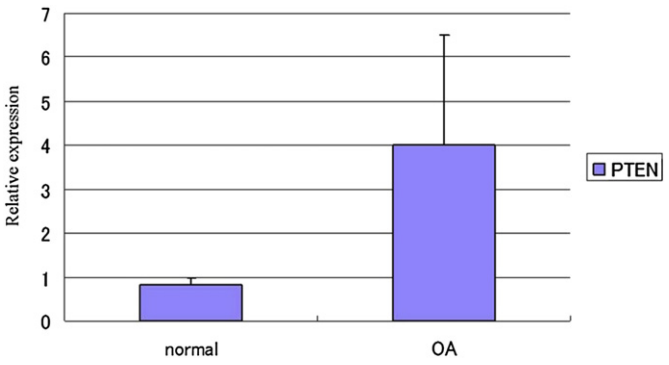

Figure 1. PTEN expressions of hip normal, hip OA and knee OA.

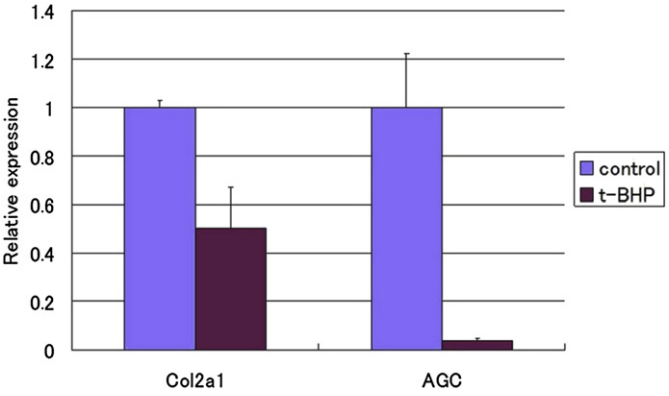

Figure 2. Col2a1 and AGC expressions after treated with t-BHP Normal chondrocytes.

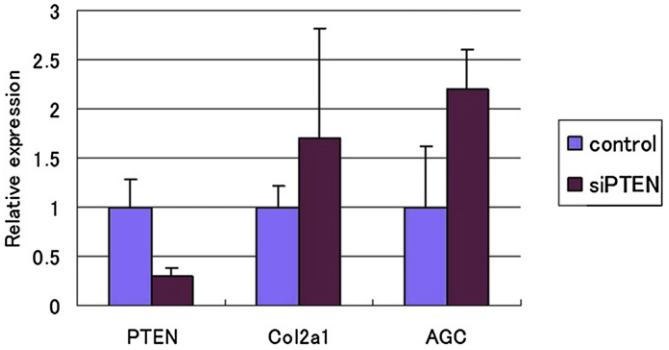

Figure 3. PTEN, Col2a1 and AGC expressions after treated with t-BHP and transfection of PTEN siRNA Normal chondrocytes.